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Highly efficient purification of porcine diamine oxidase

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Abstract

Diamine oxidase (DAO) is a member of the class of copper-containing amine oxidases and catalyzes the oxidative deamination of histamine and other biogenic amines. The enzyme from porcine kidney was purified by consecutive chromatography on concanavalin A Sepharose, heparin Sepharose and Mono Q. Besides being simpler and faster than previous methods, this new purification scheme results in a homogenous product with a considerably higher yield and allows the rapid purification of large amounts of DAO from mammalian tissues. The availability of sufficient pure protein will greatly facilitate future studies of the structure and function of the enzyme.

Keywords: Purification; Enzymes; Diamine oxidase

1. Introduction

Diamine oxidase (DAO, EC 1.4.3.6) degrades histamine and other biogenic amines [1]. The enzyme belongs to the class of copper-containing amine oxidases which is characterized by possessing the active-site cofactor topa quinone, formed post-translationally by modification of a conserved tyrosine residue [2–4]. These enzymes catalyze the oxidative deamination of primary amines by dioxygen to form the corresponding aldehydes, ammonia and hydrogen peroxide [5].

Copper amine oxidases converting various amine substrates are found in animals, plants and microorganisms [5]. Analyses of genes and cDNAs encoding copper amine oxidases revealed that all members of this enzyme family have homologous sequences with several absolutely conserved amino acid residues [6]. The conserved residues appear to be important for the overall protein structure and for the catalytic function and include the tyrosine that is converted to topa quinone [3,4], three histidine residues that bind the copper ion, and an aspartic acid residue important for substrate conversion [7–10]. In mammalian tissues, copper amine oxidases comprise enzymes that prefer either monoamines or diamines as substrates [5]. Diamine oxidase was originally characterized as the enzyme degrading histamine and was therefore earlier called histaminase [1]. Although DAO appears to play an important role in histamine catabolism [1,11] the fact that the enzyme efficiently converts many diamines besides histamine [12,13] and is expressed in many tissues [14,15] suggests that it might have additional functions [16].

Mammalian diamine oxidase is a homodimeric glycoprotein with subunits of a relative molecular mass of approximately 100 000 linked by disulfide

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bonds [13,17,18]. DAO primary structures are highly conserved in mammals [19–21]. DAO possesses a classical signal peptide [19,22] and is *N*-glycosylated [13] indicating that the protein enters the secretory pathway. DAO is a soluble protein mainly found inside cells. In porcine kidney and intestine, DAO is localized in vesicular structures in proximity to the plasma membrane [23]. DAO can be released into the bloodstream induced by heparin [24] and into the lymph induced by certain nutrients [25] but the significance of these phenomena is not clear.

Recent crystallographic studies of copper amine oxidases from microorganisms and plants [7–10] have enormously contributed to the understanding of the structural organization and enzymology of these proteins. Unfortunately, mammalian family members have so far not been available for such analyses mainly because mammalian copper amine oxidases are difficult to purify to homogeneity in sufficient quantity. Here we describe a new purification scheme that exploits the strong binding of porcine diamine oxidase to concanavalin A and to heparin and that allows the rapid purification of large amounts of homogenous protein.

2. Experimental

2.1. Materials and equipment

Putrescine (1,4-diaminobutane) was from Merck (Darmstadt, Germany). [^{14}C]Putrescine (1,4-diamino-[1,4-^{14}C]butane dihydrochloride) (118 Ci/mol) was purchased from Amersham (Buckinghamshire, UK). Bulk concanavalin A Sepharose, prepacked HiTrap Heparin and Mono Q HR5/5 columns, all chromatographic equipment and a fast protein liquid chromatography (FPLC) system were obtained from Pharmacia (Uppsala, Sweden). Dialysis tubing of regenerated cellulose with a molecular mass cutoff of 14 000 was obtained from Roth (Karlsruhe, Germany). In order to minimize protein adsorption especially of dilute fractions the tubing was soaked in 0.01% Tween 20 for 60 min followed by extensive washes in distilled water. All other chemicals used in this study were of analytical grade.

2.2. Purification of diamine oxidase

Porcine kidney cortex tissue was obtained immediately post mortem, cut into small pieces, immediately frozen in liquid nitrogen, and stored at −75°C until use. A 30-g amount of frozen tissue was thawed on ice and homogenized in three volumes of 20 m*M* bis-Tris-hydrochloride, pH 7.0 containing 1 m*M* phenylmethanesulfonyl fluoride (PMSF) for 6 min using an Ultra-Turrax T25 from Janke and Kunkel (Staufen, Germany) with a S25N-25GM probe at 10 000 rpm. The homogenate was centrifuged at 5000 *g* at 4°C for 10 min and the pellet was rehomogenized for 4 min in one volume of the same buffer and spun again at 5000 *g*. The supernatants were combined and cleared by consecutive centrifugation at 30 000 *g* at 4°C for 10 min and at 48 000 *g* at 4°C for 20 min. The final supernatant was dialyzed against 50 volumes of 20 m*M* bis-Tris-hydrochloride, pH 7.0 containing 0.1 m*M* PMSF at 4°C.

All chromatographic steps were performed on a FPLC system from Pharmacia at room temperature and fractions were immediately chilled and stored at 4°C. The dialyzed homogenate was adjusted to 500 m*M* NaCl and loaded on a concanavalin A Sepharose column (D=1.6 cm, L=5 cm, V=10 ml, flow=1.5 ml/min). The column was washed with 10 volumes of 20 m*M* bis-Tris-hydrochloride, pH 7.0-500 m*M* NaCl and bound proteins were eluted with the same buffer containing 500 m*M* methyl-α-D-mannopyranoside. The eluate containing DAO was dialyzed against 50 volumes of 20 m*M* bis-Tris-hydrochloride, pH 7.0 at 4°C and was then loaded on a HiTrap heparin Sepharose column (D=1.6 cm, L=2.5 cm, V=5 ml, flow=2.5 ml/min). After washing the column with 10 volumes of 20 m*M* bis-Tris-hydrochloride, pH 7.0 weakly bound proteins were removed by elution with the same buffer containing 250 m*M* NaCl. DAO which strongly binds to the heparin Sepharose was then eluted with a linear gradient of 250–1000 m*M* NaCl in 25 ml 20 m*M* bis-Tris-hydrochloride, pH 7.0. DAO-containing fractions were combined and dialyzed against 50 volumes of 20 m*M* bis-Tris-hydrochloride, pH 7.0 at 4°C. The dialyzed heparin Sepharose fractions were loaded on a Mono Q HR5/5 column (D=0.5 cm, L=5 cm, V=1 ml, flow=1 ml/min). After washing with five volumes of 20 m*M* bis-Tris-hydrochloride,

pH 7.0 bound proteins were eluted with a linear gradient of 0–400 m*M* NaCl in 20 ml 20 m*M* bis-Tris-hydrochloride, pH 7.0. DAO eluted as a single peak at 150–200 m*M* NaCl. DAO-containing fractions were combined and stored at 4°C. The purified protein was stable and no decrease of the enzymatic activity was observed.

2.3. Protein analyses

Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [26]. Samples were electrophoresed in the presence of 240 m*M* 2-mercaptoethanol in 7.5% gels and proteins were visualized by silver-staining. Protein concentrations were determined according to Bradford [27] with bovine plasma gamma globulin as standard using a kit from Bio-Rad (Vienna, Austria). DAO activities were determined by a radiometric microassay based on the conversion of [^{14}C]putrescine [28]. Briefly, the sample is incubated in a total volume of 100 μl in 100 m*M* sodium phosphate, pH 7.4 containing 0.01% Tween 20 with 10 nCi [^{14}C]putrescine (final concentration 0.45 m*M*) at 37°C. Addition of Tween 20 does not alter the activity but is essential for pure and dilute fractions of DAO to prevent adsorption of the protein to the tube walls. The reaction is terminated by addition of 10 μl 10% perchloric acid followed by alkalization with 50 μl 600 m*M* sodium carbonate, pH 12.2. The reaction product, [^{14}C]Δ_1-pyrroline, is extracted with 10 volumes of toluene containing 0.35% 2,5-diphenyloxazole and its radioactivity determined by liquid scintillation counting. The activity is calculated in international units (U) where one unit converts 1 μmol of putrescine per minute at 37°C.

3. Results

Due to the relatively high expression level of diamine oxidase in porcine kidney cortex [15] and because of our previous experience in the purification and characterization of porcine kidney DAO [13] we chose this tissue as starting material to develop a highly efficient purification scheme for this protein. The new procedure takes advantage of several properties of DAO concerning protein stability, specific binding properties, and conservation of enzymatic activity. As described in detail in the Section 2.2 the tissue material was mechanically homogenized, the homogenate was cleared by centrifugation at 48 000 *g* and dialyzed. The dialyzed 48 000 *g* supernatant was purified in three chromatographic steps to obtain a homogenous product consisting of DAO.

The elution profiles of the chromatographic separations are shown in Fig. 1 and the summary of the purification is presented in Table 1. Due to its glycoprotein nature DAO strongly binds to Concanavalin A Sepharose and can be eluted with high concentrations of methyl-α-D-mannopyranoside (Fig. 1A). Bound glycoproteins were eluted in a single large fraction since application of a flat gradient of methyl-α-D-mannopyranoside did not result in an appreciable separation of DAO from other bound proteins. This step led to a ca. 40-fold enrichment of DAO without any loss of activity.

The strong binding of DAO to heparin was exploited in the second step (Fig. 1B). Removal of weakly bound proteins by a step gradient of 250 m*M* NaCl prior to elution of DAO with a linear gradient of 250–1000 m*M* NaCl led to a considerably purer product in the DAO-containing fractions. The loss of 50% of activity in this step might possibly be explained by the interaction with heparin since we found that some preparations of heparin can inactivate DAO in vitro (D.W. and H.G.S., unpublished results). The final polishing step by anion-exchange chromatography of the heparin Sepharose fractions on Mono Q (Fig. 1C) removed several minor impurities with only minimal loss of DAO activity.

Starting with 30 g pig kidney cortex tissue a total of 2.4 mg homogenous DAO was purified 545-fold with 44% yield (Table 1). An analysis of the proteins in individual fractions by SDS-PAGE is shown in Fig. 2. DAO migrates at am M_r of 104 000 and forms a single fuzzy band due to its heterogenous glycosylation [13].

4. Discussion

Earlier studies describing the purification of porcine kidney DAO [29,30] are difficult to compare to

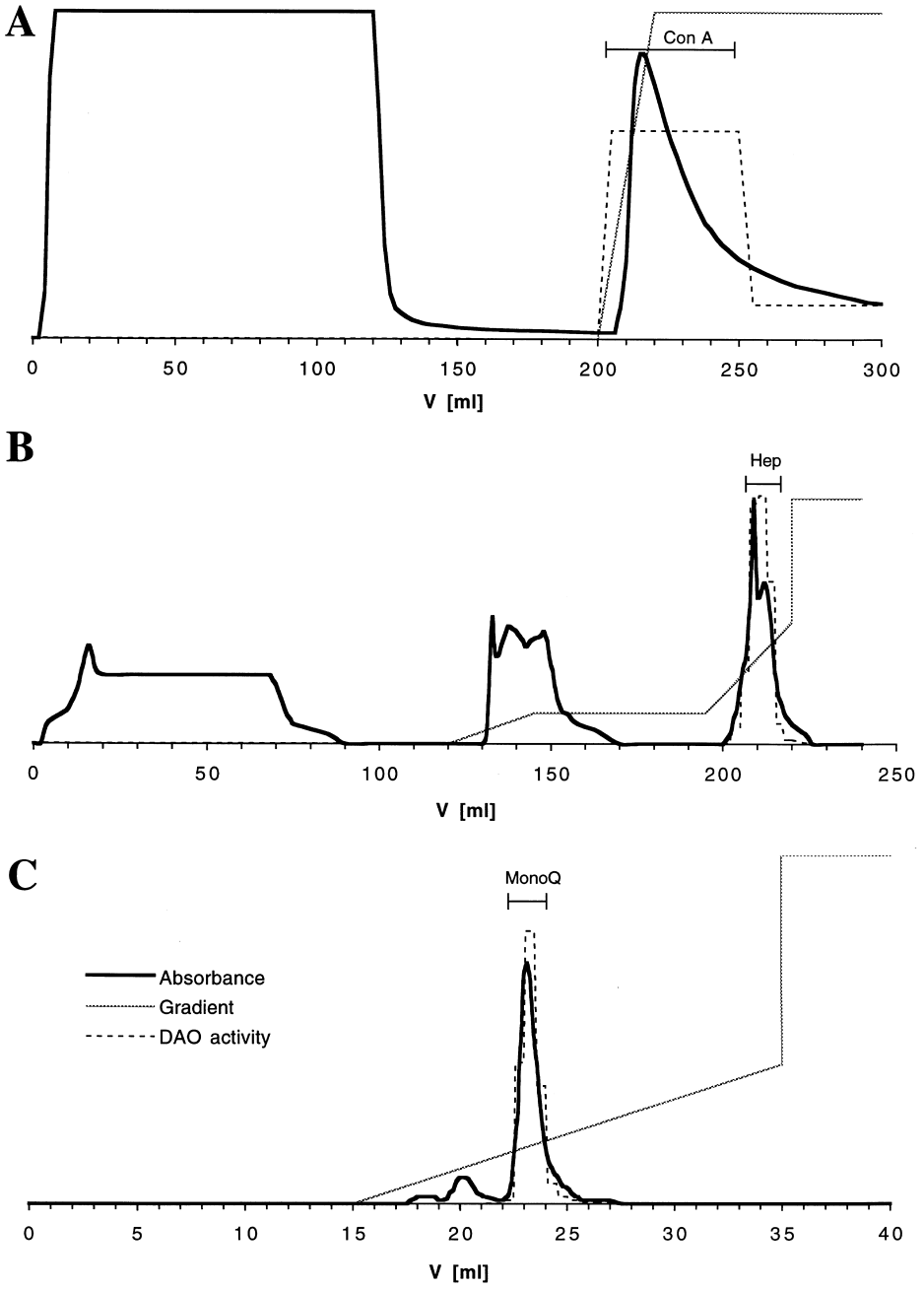

Fig. 1. Chromatograms of the three purification steps. Absorbance values at 278 nm, DAO activities and gradient shape are plotted against elution volumes. DAO-containing fractions that were combined are indicated by horizontal bars and are labeled Con A, Hep and Mono Q, respectively. (A) Concanavalin A Sepharose chromatography of the homogenate. Full scale corresponds to 2.0 absorbance units, 250 mU/ml DAO activity, and 500 m*M* methyl-α-D-mannopyranoside, respectively. (B) Heparin Sepharose chromatography of the combined concanavalin A Sepharose fractions. Full scale corresponds to 2.0 absorbance units, 450 mU/ml DAO activity, and 2 *M* NaCl, respectively. (C) Mono Q chromatography of the combined heparin Sepharose fractions. Full scale corresponds to 1.0 absorbance units, 3500 mU/ml DAO activity, and 1 *M* NaCl, respectively.

Table 1
Summary of the purification of diamine oxidase from porcine kidney[a]

Fraction	P (mg)	A_T (mU)	A_S (mU/mg)	PF (fold)	Yield (%)
Homogenate	2990	7322	2.5	1	100
Con A Sepharose	80	7454	93	38	102
Heparin Sepharose	5.5	3670	667	272	50
Mono Q	2.4	3209	1335	545	44

[a] The table summarizes the amount of protein (P), the total enzymatic activities (A_T), the specific enzymatic activities (A_S), the purification factor (PF), and the yield of diamine oxidase in the various fractions. DAO activities were determined with [^{14}C]putrescine as substrate and 1 U corresponds to the conversion of 1 μmol per min at 37°C.

our present work because different, less sensitive assay methods were used to determine the enzymatic activity and no evidence was provided for the homogeneity of the products. Although comparable specific activities were obtained for the purified proteins, the ca. five-fold lower activities in the homogenates might explain the high purification factors and yields in these studies. The 545-fold purification obtained here corresponds well with DAO constituting 0.1–0.2% of the total soluble protein of porcine kidney cortex homogenates estimated from immunoblotting experiments (H.G.S., unpublished results). Compared to our previous description of the purification of DAO from porcine kidney [13] significantly higher purification factors and yields were obtained in the present study. Besides optimization of concanavalin A Sepharose and Mono Q chromatography, the major improvement was the replacement of hydrophobic interaction chromatography on phenyl Sepharose by affinity chromatography on heparin Sepharose as the second step. Purification of porcine DAO by chromatography on heparin-agarose was described earlier [31] but the procedure was used for a crude lysate and gave a low yield. It appears to be critical to perform heparin Sepharose chromatography rapidly to avoid the possible inactivation of DAO by prolonged interaction with this ligand.

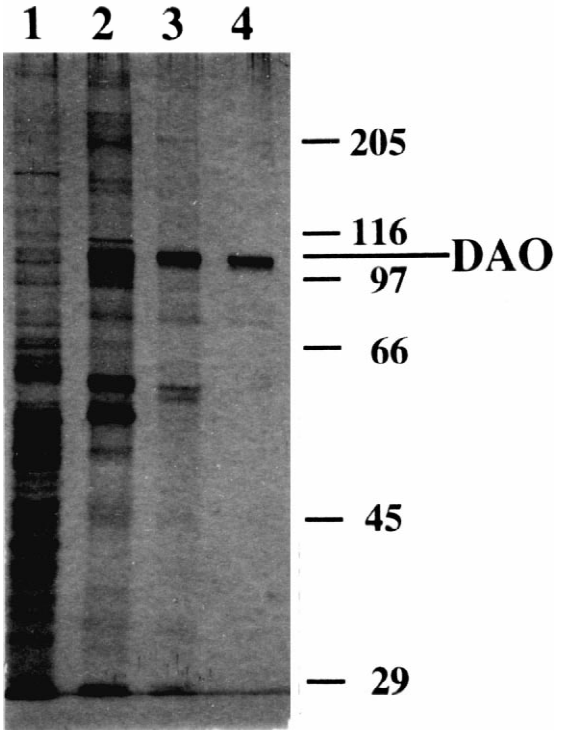

Fig. 2. Gel electrophoretic analysis of chromatographic fractions. Proteins of the homogenate (lane 1), the concanavalin A Sepharose fraction (lane 2), the heparin Sepharose fraction (lane 3), and the final Mono Q fraction (lane 4) were separated on a 7.5% SDS polyacrylamide gel under reducing conditions. Proteins were silver-stained. The migration positions of molecular mass markers (values given are $M_r \cdot 10^{-3}$) and of diamine oxidase (DAO) are indicated on the right.

In summary, the combination of two affinity chromatography and one high-resolution anion-exchange chromatography steps allowed a highly efficient purification of diamine oxidase from a porcine kidney homogenate. The new purification scheme is simpler and faster than previous methods and yields large amounts of homogenous DAO. The availability of sufficient pure protein will greatly facilitate future studies to elucidate the structure of this enzyme and its function in various metabolic pathways in mammalian cells.

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